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Larger is better: The effects of floral display on reproductive success in two populations of *Caladenia (Stegostyla) gracilis* R. Br.

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Abstract

Floral display can affect pollinator visitation and reproductive success. If floral display is genetically regulated and if pollinators preferentially visit some types of displays more frequently than others, then there are opportunities for selection. The effect of variable numbers of flowers in the terrestrial orchid *Caladenia (Stegostyla) gracilis* R.Br. on reproductive fitness was investigated in two populations. Fitness measures included the number of male (pollinaria removal) and female (pollinaria deposition and fruit set) reproductive successes. In both populations studied, all three fitness indices demonstrated the same trends that inflorescences with a greater number of flowers have a higher probability of having pollinaria removed, deposited and of producing fruits. This trend of increased reproductive success of plants with larger inflorescences was not expected in this mostly few-flowered clade, the Caladeniinae. If no other counter selective fitness measures are present, then inflorescences with greater number of flowers in *C. gracilis* can be expected in future generations, however, the increased fitness advantage for larger displays may be mediated by a reduction in lifetime fitness or survival of larger individuals, moreover flower number may be a response to phenotypic plasticity to environmental resources and may not be easily inherited if at all.

Introduction

Evolution is the result of a combination of preferential (natural selection) and random (genetic drift) changes among heritable variable characters in a species across generations (Endler, 1986). Morphological and genetic variations in a species are the potential source of evolutionary activity. Many species of plants have variable floral display and consequently there is an opportunity for pollinator directed selection (Williams and Conner, 2001; Kobayashi *et al.*, 1997; Worley *et al.*, 2000; Totland *et al.*, 1998).

The evolution of floral display has been studied in a number of species starting with *Asclepia* (Willson and Ratchke, 1974; Willson and Price, 1977). There has been an extensive interest in the evolution of floral display in many plants (Schemske, 1980; Kobayashi *et al.*, 1997). Orchids have been viewed as the ultimate evidence of pollinator driven evolution by many authors, starting with Darwin (1877). The floral morphology of orchids and the intricate interactions with their pollinator(s) have been described by Darwin (1877) and others for many orchid species and it is expected that floral display (number of flowers on an inflorescence) is equally driven by pollinators. Pollinator responses (positively or negatively) to floral display, morphology and colour in natural field populations have been evaluated in a number of orchids (Schemske, 1980; Gigord *et al.*, 2001; Maad, 2000; O'Connell and Johnston, 1998; see Tremblay *et al.*, 2005 for an extensive review). In an early orchid study evaluating pollinator-mediated selection, Schemske (1980) demonstrated that pollinators of *Brassovola nodosa* are preferentially attracted to larger inflorescences. The pattern observed by Schemske (1980) is not unique and has been corroborated in other studies (Montalvo and Ackerman, 1987; Aragón and Ackerman, 2001), but the pattern is not consistent for all orchid species (Meléndez-Ackerman and Ackerman, 2001; Flores-Palacios and Garcia-Franco, 2003).

In this study, the effect of pollinator-mediated selection in the terrestrial orchid, *Caladenia gracilis* R. Br. was investigated in two Victorian populations with inflorescences of one to four simultaneously open flowers. The strength of phenotypic selection was indicated as relative measures of male and female reproductive success.

Methods

MODEL SYSTEM

Caladeniinae is a large group of Australian terrestrial orchid taxa that recently received intensive taxonomic and systematic attention (Hopper and Brown, 2000; 2001; Jones *et al.*, 2001; Clements *et al.*, 2002) resulting in a much narrower circumscription of *Caladenia*. Most of the taxa in the Caladeniinae are few-flowered with one to six flowers per inflorescence (Backhouse and Jeanes, 1995), however the smaller inflorescences are most common and uni-flowered inflorescences are present in all of the species. Adding the floral display size of the different species of *Caladenia* studied by Jones *et al.* (2001) to their phylogenetic tree suggest that the evolutionary direction of floral display size across *Caladenia* s.l. does not show any clear patterns of increase or decrease (data not shown). However the information on display size is less than satisfactory as in the species description the range of flower size is given and not its relative importance.

Caladenia gracilis is a widespread endemic species to eastern Australia, including Victoria, South Australia, Tasmania, New South Wales, ACT and Queensland (Backhouse and Jeanes, 1995). Plants are usually found in poor soils in open woodlands and heaths (Backhouse and Jeanes, 1995). Flowers are small, 30 mm across on an inflorescence up to 45 cm tall. Bishop (1996) mentions that plants can have up to 6 flowers although none that size were seen in this survey.

SITES

A total of 81 plants was surveyed from two sites on the 19th October 2004. Thirty-nine plants from Boomers Nature Conservation Reserve (37° 38"S 145° 16"E) and 42 plants from One Tree Hill Nature Conservation Reserve (near Bendigo, Victoria; 36° 48"S 144° 19"E) were surveyed for pollinaria deposition, removal and fruit set. The selection of plants follows a non- random survey along both sides of the pathways at both sites.

STATISTICS

Total reproductive success of *Caladenia gracilis* at the species level and for each site was evaluated from percent pollinaria removal, deposition and fruit set. Differences within and among sites were tested with Kruskal-Wallis non-parametric test. Logistic regressions were used to evaluate if plants with larger floral display have preferential reproductive success through male fitness (pollinaria removal: PR) or female fitness (pollinaria deposition: PD and fruit set: FR). Population level reproductive success was calculated as the total pollinaria removed, pollinaria deposited and fruit set over total flowers surveyed. To evaluate the selective advantage of differential floral display three relative fitness indices were calculated for each individual. These indices compare the individual fitness to the mean population fitness as a function of pollinaria removal (PR), $Fitness_{PR} = \frac{PR}{Mean\ (PR)}$, pollinaria deposition on stigma (PD), $Fitness_{PD} = \frac{PD}{Mean\ (PD)}$,

and fruit set . $Fitness_{PR} = \frac{PR}{Mean\ (PR)}$ Relative fitness is a measure of the contribution of the specific variable trait to the next generation as compared to the other variables and is the preferential method for estimating fitness differences among phenotypes (Lande and Arnold, 1983; Endler, 1986; Bennington and McGraw 1995).

Results

SPECIES LEVEL REPRODUCTIVE SUCCESS

A total of 160 flowers from 81 individuals was examined of which 40 flowers had their pollinaria removed (25%), while only 21 flowers were observed with pollinaria deposited on the stigma (13.1%) and 24 fruits were observed (15%). Assuming that all flowers with pollinaria deposited produce viable fruits and all fruits attain maturity, a total of 45 flowers would produce fruits, bringing fruit set to 28.1%.

FREQUENCY OF FLORAL DISPLAY SIZE

Display size of four flowers on an inflorescence was uncommon (5%) while most plants had only one (27.2%) or two flowers (53.1%;). The frequency distribution of floral display per plant was not significantly dependent on the site (Log Likelihood 2.19, df, 3, 75, p = 0.22; Fig. 1).

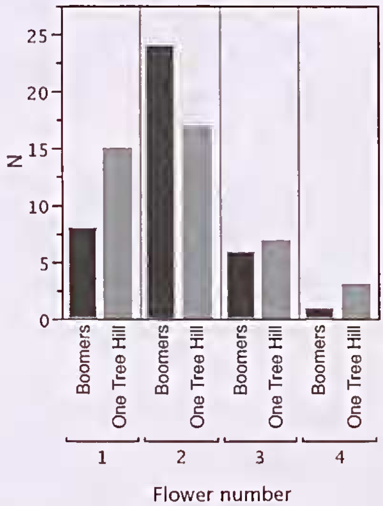

Figure 1: Frequency distribution of floral display in 81 individuals at two Victorian sites, One Tree Hill and Boomers Nature Conservation Reserves. N = number of individuals.

POPULATION VARIATION IN REPRODUCTIVE SUCCESS

The orchids surveyed at Boomers Nature Conservation Reserve and One Tree Hill Nature Conservation Reserve had significantly different levels of pollinaria removal, deposition and fruit set. However, the pattern was not consistent between sites. Pollinaria removal and deposition was higher at One Tree Hill Reserve while fruit set was higher at Boomers Reserve (Table 1).

Table 1: The number of plants and flowers surveyed, mean flowers per plant, mean pollinaria removal, deposition and fruit set at Boomers and One Tree Hill Nature Conservation Reserves. All tests performed with non-parametric Kruskall-Wallis test. NS = not significant.

	Boomers Reserve	One Tree Hill	Test of Significance
Number of plants	39	42	
Number of flowers	78	82	
Mean number of flowers per plant ± s.e.	2.0 ± 0.11	1.95 ± 0.14	NS
Mean number of pollinaria removed per plant ± s.e. (Number of male success)	0.28 ± 0.09 (11)	0.71 ± 0.15 (29)	Z = 2.32, p = 0.02
Mean number of pollinaria deposited per plant ± s.e. (Number of female success)	None (0)	0.51 ± 0.12 (21)	Z = 4.14, p < 0.0001
Mean number of fruits per plant ± s.e. (Number of female success)	0.49 ± 0.13 (19)	0.12 ± 0.05 (5)	Z = 2.44, p = 0.014

DISPLAY SIZE AND REPRODUCTIVE SUCCESS

The reproductive success through male and female indices is influenced by the display size. Plants with fewer flowers were significantly less likely to get effectively visited, either through pollinaria removal, pollinaria deposition or fruit production (Table 2). All three reproductive indicators showed a substantial increase with larger floral display. The effect of floral display was especially evident when pollinaria removal was considered; a plant with one flower had less than 10% probability of having the pollinaria removed while a plant with three flowers had close to 80% of the flowers with pollinaria removed. The reproductive indices of pollinaria deposition and fruit set also showed that a small display sizes are less effective in attracting pollen and setting fruits (Table 2).

Table 2: The observed mean ± standard error of pollinaria removed (PR), pollinaria deposited (PD) and fruits set (FR) per plant in *Caladenia gracilis* varying in number of flowers per plants. Relative fitness (RF) measure is an index of realized proportional number of PR, PD and FR success as compared to the mean fitness of the population.

Number of flowers (Sample size)	Mean PR	Mean PD	Mean FR	RF (PR)	RF (PD)	RF (FR)
1 (22)	0.09 ± 0.14	0.09 ± 0.29	0.09 ± 0.29	0.18 ± 0.13	0.35 ± 0.24	0.29 ± 0.21
2 (43)	0.44 ± 0.01	0.20 ± 0.46	0.20 ± 0.40	0.93 ± 0.21	0.74 ± 0.27	0.66 ± 0.21
3 (14)	0.79 ± 0.17	0.54 ± 0.88	0.92 ± 1.04	1.54 ± 0.46	2.05 ± 0.93	3.12 ± 0.97
4 (4)	2.25 ± 0.33	1.00 ± 1.41	0.50 ± 1.00	4.50 ± 1.26	3.81 ± 2.69	1.69 ± 1.69

Display size positively affected male reproductive success, with larger displays having had significantly greater proportion of pollinaria removed (Logistic regression; log Likelihood = 8.134, df = 1, 80, $p < 0.0001$, R^2= 15.7%), while both female reproductive success indices showed a positive response with display size, however, these models explained less of the variation (Pollinaria deposition, Logistic regression; log Likelihood = 2.37, df = 1, 80, $p < 0.03$, R^2= 6.1%; Fruit set, Logistic regression; log Likelihood = 2.998, df = 1, 80, $p < 0.01$, R^2= 7.0%). Plants with a smaller display size (1 flower per inflorescence) are expected to have only about 10% reproductive success (for male and female success), while 30% of those with a display size of three or more flowers, should have female reproductive success and more than 60% are expected to have male reproductive success (Table 3).

Table 3: The expected male and female reproductive success of individuals of *Caladenia gracilis* with varying floral display. Analysis of receiver operating characteristics (ROC) calculates the most likely state of each of the type of floral display from the logistic regression equation. Thus a flower on a two-flowered inflorescence has a 32% chance of having the pollinaria removed, while on a four-flowered inflorescence, an individual flower has an 89% chance of having the pollinaria removed.

Floral display (number of flowers)	Expected percent pollinaria removal	Expected percent pollinaria deposition	Expected percent Fruit set
1	0.11	0.09	0.10
2	0.32	0.17	0.21
3	0.66	0.30	0.37
4	0.89	0.47	0.57

Table 4: Effect of floral display on reproductive success in orchids. "+" = positive effect of larger floral display on reproductive success, "-" = negative effect of larger floral display on reproductive success, NS = no significant effect of floral display on reproductive success.

Species	Variation in number of flowers	Pollinaria removal	Fruit set	References
Aspasia principissa	1-7	+	+	Zimmerman and Aide, 1989
Brassavola nodosa	1-5	+	+	Schemske, 1980; Murren and Ellisson, 1996
Calopogon tuberosus	1-10		NS	Firmage and Cole, 1988
Comparettia falcata	1-9	+	+	Rodríguez *et al.*, 1992
Cyclopogon cranichoides	8 - > 40		+	Calvo, 1990
Dactylorhiza maculata	Mean 15		NS	Vallius, 2000
Encyclia krugii	1-8	NS	NS	Ackerman, 1989
Epidendrum exasperatum	6-358	+		Calvo, 1990
Epipactis helleborine	15-30? data absent from paper	+	+	Ehlers, *et al.*, 2002
Ionopsis utricularioides	1-44	-	-	Montalvo and Ackerman, 1987
Lepanthes wendlandii	1-123		NS	Calvo, 1990
Oeceoclades maculata	4-16		NS	Calvo, 1990
Platanthera bifolia	10-20	+	+	Maad, 2000

Discussion

Floral display in *C. gracilis* is skewed towards few flowers per individual and plants with more flowers have significantly greater reproductive success as measured from pollinaria removal, pollinaria deposition or fruit set. If floral displays were controlled only by genetics, and resources and the environment had no effect or interaction with genetics (phenotypic plasticity), we would expect a sufficiently fast rate of evolution to large inflorescences within a few generations. However, inflorescence size is likely to be at least partially controlled by nutrient and water availability and vary with site and temporal variations in biotic and abiotic resources.

The increase in reproductive success with larger flower display is not present in all orchids. In the Australian *Elythranthera brunonis* (Endl.) A.S. George no effect of single flowered versus multi-flowered individuals on reproductive success (PR and PD) was noted (Tremblay *et al.* unpublished data).

INFLUENCES ON INFLORESCENCE SIZE

Inflorescence size may be controlled by multiple factors over ecological and evolutionary time. Total flower production is dependent on plant size in many species (Harper, 1977) including orchids (Montalvo and Ackerman, 1987; Zimmerman and Aide, 1989; Calvo, 1990). Larger inflorescences including larger displays in orchids generally have greater male and female reproductive success. Moreover, plant size distribution in many populations is strongly hierarchical, with the majority of the individuals being small (Weiner and Solbrig, 1984; Gregg, 1991; Leeson, *et al.*, 1991; Montalvo and Ackerman, 1987). Consequently, most orchids have relatively small inflorescences (e.g., Schemske, 1980; Firmage and Cole, 1988, Tremblay and Ackerman, 2001) as has been shown here in *C. gracilis*. Even if larger inflorescences are beneficial, an increase in the mean inflorescence size of a population may be limited by energetic and allometric constraints. What the genetic constraints to floral display are is not evident, and what the importance of phenotypic plasticity in orchids still needs much study.

Alternatively, evidence is known that larger individuals frequently have better quality offspring (Roach and Wulff, 1987; Rossiter 1996; Weiner *et al.*, 1997). However, the effect of display size is not consistent among plant species, an experimental manipulation of inflorescence size (number of flowers) of varying fitness measures in *Ipomopsis aggregata* showed that although larger displays did attract more visits, they found no evidence that there was a proportional increase in visitation rates and found no effect on seed production (Brody and Mitchell 1997). The effect of maternal quality can have long lasting effect on to final plant size and probability of survival of the progeny (Simons and Johnston 2000).

SELF-INCOMPATIBILITY AND INBREEDING

Another factor that may impose limits to inflorescence size is the frequency of geitonogamous pollinations (Ackerman, 1989), which may be greater in larger inflorescences and result in higher rates of flower and fruit abortion due to self-incompatibility or inbreeding (e.g., Wyatt, 1982; Hessing, 1988; Klinkhamer and de Jong, 1993). Thus increased geitonogamy may result in inbreeding depression, which could counteract the positive effect of larger floral displays (increased fruit set).

COST OF FLOWERING AND REPRODUCTION

The selection advantage for larger displays in *C. gracilis* would suggest evolution towards larger inflorescences, however constraints to floral display may limit selection. If there is a cost to producing larger inflorescences or more fruits, the total lifetime reproductive success of larger plants may be less than smaller individuals if larger plants have higher probability of mortality. In orchids the cost of reproduction is almost always observed among reproductive bouts, in that in the following year plants are frequently smaller (see Tremblay *et al.* 2005 for a review). In terrestrial orchids plants may stay dormant and not emerge in the subsequent season(s) after expending energy for reproduction. For example, in the Australian *Prasophyllum correctum* D. L. Jones, flowering individuals have a 45% chance of going dormant (non-emergent) after a flowering year and only 15-20% chance of flowering during two subsequent years (Coates, *et al.*, in press). In the Estonian *Orchis ustulata* L. flowering individuals usually go dormant (57%) after flowering in the previous year and only 27% flower successively (Tali, 2002). In *Caladenia versicolor* G.W. Carr, a species related to *C. gracilis*, there appears to be a substantial cost to flowering as only 35% of flowering individuals in one year produce flowers the following year, while 53% come back as vegetative plants or are dormant (12%, unpublished data, R. Cousens). Thus the advantage of larger displays in one reproductive bout may result in a little or no reproductive success in the following reproductive bout, in consequence the lifetime reproductive success of individuals need to be evaluated to comprehend the importance of larger displays on reproductive success.

A literature review of some of the evidence of the effect of floral display on reproductive success in orchids suggests, that in most species, reproductive success is higher in larger inflorescences. Only in one species has significant negative selection been noted (*Ionopsis utricularioides*, Montalvo and Ackerman, 1987). However, in a number of orchid species pollinators appear to have no preference for floral display size (Table 4). The ability to detect a significant effect is sample size dependent, however there is no evidence to suggest that these non-significant results are an aberration. Moreover, the effect of reproductive success on floral display can be inconsistent among time and space (Maad, 2000; Ehlers et al, 2002).

To evaluate the potential for natural selection on floral display a number of parameters need to be evaluated. Do flowering individuals come back with the same number of flowers as previous flowering events Is the lifetime reproductive success of a large individual equal to small individuals? What is the importance of phenotypic plasticity on floral display? At present those data are generally missing from the literature for terrestrial and epiphytic orchids.

Conclusion

Larger floral display size for an inflorescence seems to have an evolutionary advantage in the orchid *C. gracilis.* If this evolutionary advantage has been present prior to this study, then why are larger inflorescences not more common? Constraints to display size and its evolution must be present in this system and could be influenced by complex heritability, inbreeding depression, costs to reproduction and phenotypic plasticity.

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